CHAPTER 5

INFLUENCE OF SEXUAL REPRODUCTION ON THE *FUSARIUM CIRCINATUM* POPULATION IN SOUTH AFRICA

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ABSTRACT

Fusarium circinatum (=*F. subglutinans* f. sp. *pini*) is an important pathogen in pine nurseries in South Africa. The initial outbreak of pitch canker disease caused by this pathogen was limited to a single nursery. Subsequently, several other pine nurseries in South Africa have become infected. A high level of genotypic diversity was observed in the initial South African *F. circinatum* population, suggesting that sexual reproduction occurs in the fungus. Sexual crosses performed in the laboratory indicated a low frequency of hermaphrodites in the initial population, suggesting that the fungus reproduces asexually rather than sexually. The aim of this study was, therefore, to determine the influence of sexual reproduction on the *F. circinatum* population in South Africa by comparing *F. circinatum* populations collected from initial *F. circinatum* outbreak and from recent outbreaks. This was done by determining the effective population number, N_e that estimates the contribution of the sexual and asexual cycle on the population structure. The allelic diversity determined with nine polymorphic sequence characterized amplified markers and vegetative compatibility group (VCG) diversity were also used to assess the two populations. The N_e of the initial population based on the number of male and hermaphrodites was lower (N_e = 80.6-86.6%) than in the recent outbreaks (N_e = 93.0-96.0%). The allelic diversity of the initial population was also lower (0.154) than the recent population (0.231) and six new VCGs have emerged since the initial outbreak of *F. circinatum* in South Africa 10 years ago. The increase in N_e, allelic diversity and number of VCGs over the last 10 years indicated that sexual reproduction is occurring relatively frequently, even though signs of this have not been observed in the field.

INTRODUCTION

The pitch canker pathogen, *Fusarium circinatum* Nirenberg & O'Donnell (=*F. subglutinans* (Wollenweber & Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*) causes a destructive disease of pines. *F. circinatum* first appeared in South Africa in a single forestry nursery in 1990, causing a root disease of *Pinus patula* seedlings (Viljoen *et al.*, 1994). Since this initial outbreak, *F. circinatum* has spread to several other forestry nurseries causing serious root and collar rot of various *Pinus* spp. Stem cankers on mature trees typical of those found elsewhere have, however, not been observed in South Africa (Viljoen *et al.*, 1997a; Wingfield *et al.*, 1999).

The genotypic diversity of the initial *F. circinatum* population in South Africa have been determined using vegetative compatibility groups (VCGs) (Viljoen *et al.*, 1997b) and allelic diversity using nine sequence characterized polymorphic markers (Britz *et al.*, submitted (chapter 4 of this thesis)). A high level of VCG diversity (23 VCGs among 69 isolates) led Viljoen *et al.* (1997b) to suggest that sexual reproduction is occurring naturally within the South African population. However, the allelic diversity determined using sequence characterized amplified polymorphic markers indicated a relative low allelic diversity in the initial *F. circinatum* population (Britz *et al.*, submitted). Therefore, the VCG and allelic diversity data (Viljoen *et al.*, 1997b; Britz *et al.*, submitted) indicates that sexual reproduction is occurring in South Africa among closely related isolates in the South African population. This also indicate that the pathogen in South Africa has been recent introduced (Viljoen *et al.*, 1997a, b).

Fusarium circinatum and other related *Fusarium* spp. residing in distinct mating populations of *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex (Leslie, 1995; Klaasen & Nelson, 1996; Klittich *et al.*, 1997; Britz *et al.*, 1999) is heterothallic and mating is controlled by two alleles at a single mating type locus. *F. circinatum* resides in mating population H of the *G. fujikuroi* complex (Britz *et al.*, 1999). *F. circinatum* strains of opposite mating type can recombine, leading to the formation of the sexual structures (Britz *et al.*, 1998; Viljoen *et al.*, 1997a). The name, *G. circinata* Nirenberg & O'Donnell has recently been applied to the teleomorph (Nirenberg & O'Donnell, 1998).

Fusarium circinatum can reproduce both asexually and sexually and each of these cycles affects the population structure differently (Leslie & Klein, 1996; Britz *et al.*, 1998). The asexual cycle results in clonal propagation, whereas the sexual cycle results in recombination leading to new genotypes (Leslie & Klein, 1996). The effective population number (N_e) estimates the relative contribution of each cycle towards the population (Leslie & Klein, 1996). However, the numbers of strains that function as female parents usually limits the N_e (Leslie & Klein, 1996; Britz *et al.*, 1998). The initial *F. circinatum* population in South Africa had a low frequency (27%) of hermaphrodites (Britz *et al.*, 1998). If the number of hermaphrodites in the *F. circinatum* population decreases then the population in South Africa could become asexual (Britz *et al.*, 1999; 1999).

Several new outbreaks have occurred in South Africa since the initial outbreak of *F. circinatum* in 1990 (Viljoen *et al.*, 1994). The opportunity has, therefore, arisen to be able to compare the N_e of the initial *F. circinatum* population with a recently collected *F. circinatum* population. Thus enabling the determination of the influence of sexual reproduction on the population over 10 years. However, the N_e was determined at a lower incubation temperature, because Covert *et al.* (1999) found lower temperatures increase the fertility of sexual crosses of *F. circinatum* in the laboratory. Furthermore, the VCG and allelic diversity in the *F. circinatum* population collected six years after the initial outbreak of the disease in South Africa were also determined and compared with previous studies on the initial population.

MATERIALS AND METHODS

Sampling

Fusarium circinatum isolates were collected from six different forestry nurseries in South Africa (Table 1). The initial *F. circinatum* population was from a single nursery (Ngodwana) and isolates representing the recent *F. circinatum* population were collected from nurseries in the Piet Retief, Karatara, Sutherland, Tweefontein and Klipkraal areas. The initial *F. circinatum* population represents 85 *F. circinatum* isolates collected between 1990 and 1992 (Viljoen *et al.*, 1994). The recent *F. circinatum* populations consist of 74 isolates that were obtained from outbreaks between 1996 and 1998. A total of 159 *F. circinatum* isolates were examined in this study (Table 1).

Roots, root collars and stems of pine seedlings showing disease symptoms, e.g. tip die-back and needle discoloration, were immersed in 70% ethanol for 2 min. Small pieces (approximately 5 mm long) of the infected tissue were removed and plated on *Fusarium* selective medium (Nash & Snyder, 1962). Cultures were allowed to grow for 5 days at 25°C. Small agar pieces (approximately 5 mm^2) from the edges of the colonies were transferred to 90 mm diameter Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.*, 1982). Cultures were incubated at 23°C under near-ultraviolet and cool-white light with a 12 h photoperiod to stimulate culture and conidium development. Isolates identified as *F. circinatum* on CLA were purified as single conidial cultures and stored

as conidial suspensions in 15% glycerol at –70°C and are available from the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A large number of the strains also have been deposited in the culture collection of the Medical Research Council (MRC), P. O. Box 19070, Tygerberg, South Africa.

Sexual compatibility and mating type determination

All the *F. circinatum* isolates were crossed with the mating population H tester strains, MRC 6213 and MRC 7488 (Britz *et al.*, 1998; 1999). The tester strains were crossed with each other as a positive control. Crosses were made on carrot agar as described by Klittich & Leslie (1988) at 17°C. All the crosses were examined weekly and designated sterile, female-sterile or hermaphrodite (Table 1). All fertile crosses were repeated at least once. The tester strains, MRC 7488 and MRC 6213, were designated *MAT*-1 and *MAT*-2, respectively (Steenkamp *et al.*, 2000). Isolates that produced perithecia with exuding ascospores, when crossed with the tester strains were assigned to the mating type opposite to that of the tester strains (Table 1).

The effect of mating type allele frequency and female fertility on the South African *F. circinatum* population was assessed by determining the effective population number for the mating type ($N_{e(mt)}$), effective population number ($N_{e(f)}$), the average number of asexual generations per sexual generation using the equations described in Leslie & Klein (1996).

Vegetative compatibility (VC) tests

Nitrate non-utilizing (*nit*) mutants were generated on 1.5 – 2.5% chlorate agar and the *nit* mutants were identified as *nit1*, *nit3* or *nit*M based on the utilization of different nitrogen sources (Puhalla & Spieth, 1983; 1985; Correll *et al.*, 1987). Incompatibility was determined by pairing isolates in all possible combinations on minimal media for 14 days at 25°C. Heterokaryon self-incompatibility (HSI) of isolates (Correll *et al.*, 1989) was determined by pairing a *nit1* or *nit3* mutant with a *nitM* mutant of the same isolate. All parings were repeated at least once.

Each VCG was assigned a number (SA1-SA29). The most common VCGs in the *F. circinatum* population were determined by identifying the number of representatives for

each VCG. The diversity of the VC groups for isolates representing the initial and recent *F. circinatum* outbreaks was determined in two ways. First, the number of VCGs was divided by the sample size in each population (S/N). Secondly, the Shannon diversity index (H′), which provides an indication of the population structure was calculated using the equation:

$$H' = -\Sigma p_i \ln p_i$$

where p_i is the observed frequency of the VC group (genotype). If all the individuals have the same genotype then $H' = 0$, and when they all belong to a different genotype, then H' will have the highest value (Sheldon, 1969).

Allelic diversity

The allele frequency and diversity values of the initial *F. circinatum* population (determined with sequence characterized amplified polymorphic markers) were obtained from Britz *et al.* (submitted) and values of the recent population were determined in the present study (Table 3). In the present study, the allelic frequency and diversity of 32 isolates representing recent *F. circinatum* outbreaks were calculated using data of nine sequence characterized amplified polymorphic markers. The DNA extraction procedures, PCR conditions, the method of determining allele size for the 32 isolates as well as the analysis were done as described by Britz *et al.* (submitted).

Multi-locus diversity was calculated using genotype data of each isolate (at all nine sequence characterized amplified polymorphic loci) from the initial (Britz *et al.*, submitted) and recent *F. circinatum* populations, $H_M = -\Sigma g_i \ln g_i$, where g_i is the frequency of the ith multi-locus genotype and $H_{EM} = H_M / \ln S$, where S is the sample size (Sheldon, 1969; Goodwin *et al.*, 1993) (Table 3).

RESULTS

Sexual compatibility and mating type distribution

Some *F. circinatum* isolates did not produce perithecia when crossed with the mating tester strains. The percentage of sterile strains collected from the different nurseries ranged from 11-17% with the exception of 29% sterile isolates found among those collected from Piet Retief nursery. No sterile *F. circinatum* isolates were collected from

the Tweefontein and Sutherland nurseries, and the single isolate collected from Karatara nursery was also sterile. Sterile isolates were excluded from the calculation of $N_{e(mt)}$ and $N_{e(f)}$. However, the $N_{e(f)}$ was also calculated for each nursery by including the sterile strains in the calculation, as female-sterile strains (Table 1).

Reassessment of initial F. circinatum population

Of the 85 *F. circinatum* isolates collected from the initial outbreak at the Ngodwana nursery, 71 were cross fertile with one of the mating type testers (MRC 6213 and MRC 7488). The mating type distribution of the initial population had a 1:1 ratio giving a $N_{e(mt)}$ of 99%. Among the 71 fertile isolates, 33 were hermaphrodites and 38 female-sterile (Table 1). The percentage of the hermaphrodites ranged from 21 - 69% (observed value 47%), when only the fertile strains were used. The average number of asexual generations per sexual generation was estimated to range from 20-79. This variation depends on the values for the mutation rate (μ) to female sterility and for selection (θ) against hermaphrodites during the asexual generation of the life cycle which were $0.98 > \mu(1-\theta) > 0.99$. When all the strains were included, then the percentage of hermaphrodites ranged from 12 - 63% (observed value 39%) with the average number of asexual generations per sexual generation ranging from 24 to 117 depending on the values of μ and θ.

Recent F. circinatum population

The recently collected *F. circinatum* population included isolates from nurseries other than the Ngodwana nursery, where the initial population was studied. In the recently collected population consisting of 74 isolates, 42 hermaphrodites were present amongst 72 fertile strains collected from five different nurseries (Table 2). The recently collected *F. circinatum* population had a 1:2 (*MAT-1*:*MAT-2*) mating type ratio, giving a $N_{e(mt)}$ of 86.7%. The hermaphrodite frequency varied from 45 – 82% (observed value 67%) with the estimated average asexual generations ranging from 10 – 39 per sexual generation, if only fertile isolates were considered. If all strains (fertile and sterile *F. circinatum* isolates from recent outbreaks) are considered, the hermaphrodite frequency ranged from 36 – 76% (observed value 58%) with the average number of asexual generations per sexual generation ranging between 13 – 51 ($0.98 < \mu(1-\theta) < 0.99$).

Combined F. circinatum population

Of the combined population of 157 *F. circinatum* isolates (initial and recent outbreaks), 134 were cross fertile with one of the tester strains (MRC 6213 or MRC 7488). These isolates included 75 hermaphrodites and 59 female-sterile strains. The hermaphrodite frequency ranged from 32 – 75% (observed value 56%) with the average asexual generations per sexual generation ranging from 15 – 56, if only fertile isolates are considered. If all strains (fertile and sterile) were included, the hermaphrodite frequency ranges from 23 – 69% (observed value 48%) with the average asexual generations per sexual generation ranging from 18 – 73 ($0.98 < \mu(1-\theta) < 0.99$).

Vegetative compatibility tests

Chlorate-resistant *nit* mutants were produced for 125 *F. circinatum* isolates. Twenty-nine VCGs were identified amongst these isolates. Of the 29 VCGs, 23 were the same as those identified from the initial *F. circinatum* outbreak occurring in 1990 (Viljoen *et al.*, 1997b). Six new VCGs were identified from the recent *F. circinatum* outbreaks. The six new VCGs were from the Klipkraal and Sutherland nurseries (Table 2). A single sterile heterokaryon self-incompatible *F. circinatum* isolate was identified.

Three dominant VCGs, SA-8, SA-12 and SA-14, were present in the initial outbreak of *F. circinatum* and represented 43% of the isolates (Viljoen *et al.*, 1997b). In the recent outbreaks, VCG SA-2 and SA-4 were dominant and represented 43% of the isolates. All VCG SA-2 isolates were collected from the Ngodwana (initial outbreak) and Klipkraal nurseries. VCG SA-4 isolates were present in all nurseries except Ngodwana and Sutherland.

The diversity of VCGs varied among the nurseries (Table 2). The number of VCGs divided by the sample size in the initial, recent and combined population (S/N) was 0.32, 0.30 and 0.24, respectively (Table 2). In the Ngodwana nursery, most isolates belonged to a different VCG, H'=2.89. The H' value for the recent outbreaks was 2.25 and the H' value when all the outbreaks were considered was 3.02 (Table 2). The H' value becomes inaccurate as the number of isolates collected in each nursery decreases (N<7). With normalization, H_{EM} (Sheldon, 1969; Goodwin *et al.*, 1993), the recent population was 0.564 and the initial population was 0.678 (Table 2). However, most of

the H_{EM} values of populations collected in recent outbreaks were higher than the H_{EM} value of the initial outbreak. The H_{EM} value for the population collected in Klipkraal in April 1998 from *P. elliottii* was the lowest, because most of the isolates belonged to a single dominant VCG.

Allelic diversity

The allele frequency using nine polymorphic markers for the initial *F. circinatum* population was 0.154 without adjusting for sample size (Britz *et al.*, submitted, Table 3). In this study, the allele frequency for isolates representing the recent population is 0.231 without adjusting for sample size. With adjustment for sample size, the allelic diversity (H_{TR}) for the initial population is 0.163 (Britz *et al.*, submitted) while the recent population is 0.331 (Table 3). The combined allelic diversity, H_T, for all isolates thus far studied in South Africa was 0.216. There were 25 putative alleles amongst the nine polymorphic markers in the combined South Africa populations. The number of alleles at each locus ranged from two to four, with an average 3.78 alleles per locus.

The initial *F. circinatum* population had nine multi-locus genotypes and the recent population had 18 multi-locus genotypes. The combined population had 25 multi-locus genotypes. The multi-locus diversity measured using the Shannon statistic H_M, was 1.730 for the initial population and 2.680 in the recent population (Table 3). The diversity values were normalized by maximizing the possible diversity in view of the sample size in each location (Sheldon, 1969; Goodwin *et al.*, 1993). With the normalization, H_{EM}, the diversity value of the initial South African population was 0.578 and that of the recent population was 0.773 (Table 3).

DISCUSSION

The first outbreak of *F. circinatum* in South Africa was reported in a single nursery (Viljoen *et al.*, 1994). Since this time, the disease has spread to several other pine nurseries in the country. A high level of VCG diversity was reported in the initial South African population, indicating that sexual recombination is occurring (Viljoen *et al.*, 1997b). However, the sexual structures of *F. circinatum* have never been found in the field. The primary objective of this study was to consider changes in the *F. circinatum* population in South Africa since the first outbreak. In this study, the N_e and number asexual generations per sexual generation of the recent *F. circinatum* population

indicates that sexual reproduction probably occurs more frequently than was obvious when the initial population was studied. This is evident from the increase in allelic diversity and the fact that new VCGs are present in the recently collected *F. circinatum* population. All indications are that active sexual reproduction is the origin of the higher level of genotypic diversity in the recently collected populations of *F. circinatum* in South Africa.

The fact that sexual structures representing *G. circinata*, have not been observed in the field is intriguing. This could be attributed to the fact that sexual reproduction does not occur at the time that the fungus is most commonly collected during disease outbreaks. This would be consistent with the fact that sexual reproduction often is coupled to overwintering, whereas asexual reproduction is coupled to the epidemic phase of disease (Linders, 1996). In the laboratory, *F. circinatum* also requires low temperatures to undergo sexual recombination (Covert *et al.*, 1999) and it seems likely that our sampling strategy has not co-incided with the appearance of sexual structures as most of the disease out breaks have occurred during the warmer months.

The high $N_{e(f)}$ in this study indicates that sexual reproduction in the South African *F. circinatum* population is more frequent for recent outbreaks ($N_{e(f)}$ = 93.1-96.0) than it was at the time of the initial outbreak ($N_{e(f)}$ = 80.6-86.6). The allelic diversity and multi-locus diversity also support the view that sexual reproduction is more frequently associated with recent outbreaks of *F. circinatum*. However, the Shannon diversity index obtained from vegetative compatibility tests on isolates representing the initial *F. circinatum* outbreak was higher than that for the more recent outbreaks. This is despite the fact that six new VCGs were identified for recent outbreaks. This might be due to the fact that a larger amount of isolates was available for the initial disease outbreak in a single nursery than for the recent outbreaks in various nurseries.

Sexual crosses performed among isolates collected from the initial outbreak of *F. circinatum* indicated a low hermaphrodite frequency (Britz *et al.*, 1998). These authors hypothesized that the number of hermaphrodites would continue to fall due to the high number of female-sterile strains and that the population would evolve towards asexuality. However, in the present study, a lower incubation temperature was used due to the fact that it is known to promote sexual crossing in cultures of *F. circinatum*

(Covert *et al.*, 1999). Female fertility and mating type distribution for the *F. circinatum* population from the initial disease outbreak was previously determined at 22°C (Britz *et al.*, 1998). The hermaphrodite frequency and effective population number were, therefore, reassessed in this study at 17°C. In the present study, we have shown that the hypothesis of Britz *et al.* (1998) was unfounded, because female fertility was inadvertently not assessed at optimal conditions. Using optimized conditions, we have now shown that a high frequency of hermaphrodites was present in the initial *F. circinatum* population (47%). This indicates that sexual reproduction occurs frequently in this population and that the relative number of hermaphrodites is increasing as proposed by Leslie & Klein (1996).

After the initial outbreak of *F. circinatum* in a single nursery in 1990, the fungus has spread to other major pine nurseries in South Africa. The fact that the same VCGs were found in the recent outbreak situations, suggests that the fungus has been introduced to these nurseries. It is known that the fungus can move to new areas with infected seedlings (Gordon *et al.*, 1996). However, nurseries in South Africa are not known to exchange plant material. It is more likely that the fungus has moved through air or seed-borne inoculum. *F. circinatum* now appears to be well established and widely distributed in pine nurseries in South Africa. It is an aggressive pathogen and management strategies will be needed to reduce losses.

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Table 1. Mating type and fertility characteristics of the *Fusarium circinatum* isolates collected in various South African forestry nurseries.

Nurseries[a]	Host	Collection Date	Characteristics of isolates[b]									
			n	*MAT-1*	*MAT-2*	N	FS	H	S	$N_{e(mt)}$	$N_{e(f)}$	$N_{e(f)}$[c]
Ngodwana (NG)[b]	*P. patula*	Jun 1990 – Jul 1992	71	35	36	85	38	33	14	99.9	86.6	80.6
Piet Retief (PR)	*P. patula*	1996-1997	5	3	2	7	5	0	2			
Karatara (KA)	*P. radiata*	1996	-	-	-	1	-	-	1	Data not determined due		
Sutherland (SU)	*P. patula*	Sept 1998	8	0	8	8	0	8	0	to small sample size		
Tweefontein (TF)	*P. patula*	Feb 1996 & Sept 1998	2	2	0	2	2	0	0			
Klipkraal (KK1)	*P. greggii*	Mrt 1997	11	3	8	11	2	9	0	79.3	99.0	99.0
Klipkraal (KK2)	*P. patula*	Apr 1998	16	6	10	18	8	8	2	93.8	88.9	85.5
Klipkraal (KK3)	*P. elliottii*	Apr 1998	21	6	15	25	4	17	4	81.6	98.9	96.4
Total Klipkraal (KK)	-	-	48	15	33	54	14	34	6	85.9	97.1	94.8
Total recent	-	1996-1998	63	20	43	72	21	42	9	86.7	96.0	93.1
Combined populations	-	1990-1998	134	55	79	157	59	75	23	96.8	92.0	87.5

[a] Forestry nurseries with abbreviations for each. Ngodwana represents the initial *F. circinatum* population,

[b] Characteristics of *F. circinatum* isolates: n = Total number of fertile isolates as determined in crosses; *MAT-1* and *MAT-2* indicates the number of isolates belonging to either mating type; N = Total number of isolates sampled in each area; FS = Female-sterile isolates; H = Hermaphrodite isolates; S = Sterile isolates; $N_{e(mt)}$ = Effective population number based on mating type expressed as a percentage of the total amount of fertile isolates in the population; $N_{e(f)}$ = Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile isolates in each population.

[c] Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile and sterile isolates in each population.

Table 2. *Fusarium circinatum* vegetative compatibility groups in South Africa nurseries.

South African Nurseries[a]	VCGs[c]	N[d]	VCG diversity		
			S/N[e]	H'[f]	H_{EM}[g]
NG (Initial)	23	71	0.32	2.89	0.678
PR	5	7	0.71	1.55	0.797
KA	1	1	1.00	0	0
SU	1	8	0.13	0	0
TF	1	1	1.00	0	0
KK1	7	10	0.70	1.89	0.821
KK2	7	11	0.64	1.76	0.734
KK3	4	16	0.25	0.95	0.343
KK	13	37	0.35	2.10	0.582
Recent[b]	16	54	0.30	2.25	0.564
Combined	29	125	0.23	3.02	0.625

[a] Abbreviations for nurseries are listed in Table 1, with NG being the initial population and the combined population includes the initial and recent populations.

[b] Recent population collected from outbreaks since 1996-1998 in PR, KA, SU, TF and KK nurseries.

[c] Number of VCGs present in each nursery.

[d] Sample size in each nursery.

[e] Number of VCGs found in each nursery.

[f] Shannon diversity index (equation in text).

[g] Sheldons's index, $H_{EM}=H'/\ln S$, where S=sample size (Sheldon, 1969).

Table 3. Allelic, genetic and multi-locus diversity of South African *F. circinatum* populations.

South Africa population	Allelic diversity[b]	H_{TR}[c]	Multi-locus diversity		
			Genotypes	H_M[d]	H_{EM}[e]
Initial[a]	0.154	0.162	9	1.730	0.578
Recent	0.231	0.331	18	2.680	0.773
Combined	0.212	0.216	25	1.868	0.473

[a] Data obtained from Britz *et al.* (submitted (chapter 4 of this thesis)).

[b] Allelic frequency = $(1-\Sigma p_i^2)$, where p_i is the frequency of the *i*th allele (Nei, 1973) was calculated using the Microsat program.

[c] H_{TR} is the genetic diversity within each region adjusted for sample size (equation in text).

[d] The equation of Shannon statistic information, H_M (referred to as *H* by Sheldon (1969) and *M* by Goodwin *et al.* (1993)), is in the text.

[e] Sheldon's index, H_{EM} (equation in text).

CHAPTER 6

TWO NEW SPECIES OF *FUSARIUM* SECTION *LISEOLA* ASSOCIATED WITH MANGO MALFORMATION

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ABSTRACT

Mango malformation is an economically important disease of *Mangifera indica* globally. A recent DNA-based study indicated that two distinct, phylogenetic lineages previously identified as *Fusarium subglutinans* are associated with this disease in South Africa. The objective of this study was to characterize *Fusarium* isolates associated with mango malformation, including the two different *F. subglutinans* groups, based on morphological characteristics. For this purpose we examined *Fusarium* strains isolated from diseased mango inflorescences from diverse geographical origins. We also used sexual compatibility tests to determine whether sexual reproduction among the strains was possible. The morphological characters considered were; shape of the conidia, presence of mono- and/or polyphialides, origin of the conidiophores from the substrate, presence of chlamydospores and the presence of sterile coiled hyphae. Three unique *Fusarium* spp. were identified. In this paper, we provide formal descriptions for the two new taxa in the section *Liseola* that we have named *F. mangiferae* and *F. sterilihyphosum*. *F. mangiferae* is conspecific with strains that were previously identified as *F. subglutinans* and reported to be the causal agent of malformation in mango growing areas throughout the world. *F. sterilihyphosum,* on the other hand, has been isolated only from malformed mango tissue in South Africa.

INTRODUCTION

Mango (*Mangifera indica* L.) malformation is an economically important disease in mango-growing areas of the world including India, Pakistan, Egypt, South Africa, Brazil, Israel, Florida and Mexico (Kumar *et al.,* 1993; Freeman *et al.,* 1999). This disease causes abnormal development of vegetative shoots and inflorescences (Kumar *et al.,* 1993). Floral malformation is the most prominent symptom and is characterized by abnormal, thick and fleshy panicles (Varma, 1983; Kumar *et al.,* 1993). Affected panicles bear no fruit, resulting in significant economic losses (Varma *et al.,* 1974; Varma, 1983; Kumar *et al.,* 1993).

The etiology of mango malformation disease is controversial. Physiological abnormality, virus infections, mite infestations and fungal pathogens have been reported as the causal agents of this disease (Kumar *et al.,* 1993). Summanwar *et al.*

(1966) identified the fungal pathogen commonly associated with the disease as *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas (= *F. moniliforme* Sheldon var. *subglutinans* Wollenweber & Reinking), residing in section *Liseola.* Freeman *et al.* (1999) recently demonstrated that isolates identified as *F. subglutinans* induced typical mango malformation symptoms on mango trees using the isolate MRC 7559 (506/2) originally collected from mango inflorescences in Israel.

Fusarium subglutinans forms part of the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex (Leslie, 1995; Britz *et al.*, 1999). *F. subglutinans* is, however, a polyphyletic taxon (= *F. subglutinans sensu lato*) that has been associated with various plant hosts, each of which represents a distinct lineage in the *G. fujikuroi* complex (Leslie, 1995; O'Donnell *et al.*, 1998; Britz *et al.*, 1999; Steenkamp *et al.*, 1999; O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a). These lineages are difficult to distinguish using conventional morphological characters such as those proposed by Nelson *et al.* (1983). Until relatively recently, these fungi were distinguished from each other using pathogenicity and mating studies (Leslie, 1995). The different lineages representing *F. subglutinans sensu lato* are, however, readily distinguishable using DNA sequences of genes for β-tubulin, translation elongation factor EF-1α, histone *H3* and calmodulin (O'Donnell *et al.*, 1998; Steenkamp *et al.*, 1999; O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a).

Mango malformation in South Africa is associated with two phylogenetically distinct groups of isolates until recently referred to as *F. subglutinans* (Steenkamp *et al.*, 1999; 2000a). Based on the histone *H3* and β-tubulin gene sequences, one group of isolates represents a previously undescribed lineage in the *G. fujikuroi* complex. The second group of isolates is conspecific with isolates that were previously reported to be the causal agent of mango malformation (Steenkamp *et al.*, 2000a). The results presented by these authors also confirmed those of Viljoen *et al.* (1997), O'Donnell *et al.* (2000) and Leslie (personal communication), who have shown using random amplified polymorphic DNAs (RAPDs), DNA sequence of several genes and isozymes, respectively, that mango malformation is associated with two distinct species, both with morphological characters typical of *F. subglutinans.*

The aim of this study was to characterize *Fusarium* spp. isolated from malformation mango tissue, from diverse geographical origins using morphology. For this purpose the morphological characteristics proposed by Nirenberg & O'Donnell (1998) were used. Sexual compatibility tests were also used to verify the identity of some of these *Fusarium* spp.

MATERIALS AND METHODS

Morphological and cultural studies

Fusarium spp. associated with mango malformation in South Africa were isolated from mango trees in Tzaneen (Northern Province), which included the areas Letsitele (LS) and Deer Park (DP). Isolates were also collected from Nelspruit (NS), Fredenheim (FH), Malelane (ML) and Hazyview (HZ) (Mpumalanga). Other isolates used in this study were isolated from malformed mango tissue by other collectors in Florida, Egypt, Israel, Malaysia and South Africa (Table 1). Mating tester strains (MRC 6213 and MRC 7488) for *F. circinatum* Nirenberg & O'Donnell (mating population H of the *G. fujikuroi* complex) were used in sexual compatibility tests (Britz *et al.*, 1999). All the isolates were stored in 15% glycerol at –70°C in the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and the culture collection of the Medical Research Council (MRC), P. O. Box 19070, Tygerberg, South Africa.

Mango inflorescence clusters, collected in South Africa, were surface sterilized with 70% ethanol for 2 min and washed with sterile deionized water for 1 min. Single malformed flowers were removed from the sterilized cluster and plated onto a *Fusarium* selective medium (Nash & Snyder, 1962). After incubation for 5 days at 25°C, small agar pieces overgrown with mycelium were taken from the edges of the colonies and transferred to 90 mm diameter Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.*, 1982). After incubation on CLA at 25°C for 7 days single conidial isolates were prepared and stored in 15 % glycerol at –70°C.

To stimulate culture and conidial development, *Fusarium* isolates (Table 1) were transferred to CLA (Fisher *et al.*, 1982) and KCl agar (Nelson *et al.*, 1983). Cultures were incubated at 23°C under fluorescent and cool-white light with a 12 h photoperiod. After 10 to 14 days of incubation, the following morphological characters were examined: shape of the conidia, presence of mono- and/or polyphialides, origin of the conidiophores from the substrate, presence of chlamydospores and sterile coiled hyphae (Nirenberg & O'Donnell, 1998). Secondary characteristics such as growth rate and colony color (Rayner, 1970) were determined on potato dextrose agar (PDA) after incubation at 25°C in the dark (Nelson *et al.*, 1983) for the two newly described species. Each isolate was plated onto three different PDA plates and the growth rate was determined over a period of 10 days. This entire procedure was repeated once more. A one way ANOVA was done to determine whether growth rate differed significantly for the different isolates. Colony color was determined after 14 days using the colour coding system of Rayner (1970). Fifty measurements were made of all diagnostic morphological characters. The measurements are indicated as minimum, mean and maximum with the standard deviations in parentheses.

Mating type and sexual compatibility tests

The mating type (*MAT*-1 or *MAT*-2) of all the isolates included in this study were determined using the PCR-based method described by Steenkamp *et al.* (2000b). Only isolates with opposite mating types were crossed within each species and between species using the method described by Klittich & Leslie (1988) with some modifications (Britz *et al.*, 1999). Covert *et al.* (1999) found that a lower incubation temperature increased sexual fertility among isolates of the *G. fujikuroi* complex (mating population H). We, therefore, used an incubation temperature of 17°C for our crosses. Sexual crosses have already been performed within and between isolates of the two new species in a previous study (Steenkamp *et al.*, 2000a), and the present study served to confirm those results using a more clearly defined collection of isolates. Since the morphological characteristics of some isolates were similar to those of *F. circinatum*, all isolates (Table 1) were crossed with the standard tester strains (MRC 6213 and MRC 7488) for *G. circinata* Nirenberg & O'Donnell (anamorph: *F. circinatum*).

RESULTS

Morphological and cultural studies

Fusarium isolates from trees suffering from mango malformation in Malaysia, Egypt, Israel, South Africa, and Florida were separated into three different groups based on morphological characters defined by Nirenberg & O'Donnell (1998). Based on the morphological characters that were used, each of these groups represented new species in the *G. fujikuroi* complex. Of the three groups, two are clearly discrete taxa based both on morphological characteristics and sequencing data (Steenkamp *et al.*, 2000a). These two taxa are represented by an extensive group of isolates and we elect to describe them as new species in *Fusarium* section *Liseola*.

Fusarium mangiferae Britz, Wingfield et Marasas sp. nov. Figs. 1-4

Coloniae in agaro PDA apud 25°C 3.4 mm per diem crescentes. Mycelium aerium floccosum, album, infra roseolo-luteum ad atropurpureum. Conidiophora in agaro CLA erecta vel prostrata, simplicia vel ramosa, cellulae conidiogenae mono- et polyphialides, usque ad 30 x 3 µm. Hyphae steriles absentes. Microconidia in capitulis falsis, hyalina, plerumque obovoidea, subinde ovata vel allantoidea, plerumque 0-septata, subinde 1-septata, (2.5–)4.3–9.0–18.4 x (1.5–)1.7–2.4–3.3 µm. Sporodochia praesentia, alba ad aurantiaca. Macroconidia hyalina, falcata, gracilia, leniter curvata, tenuitunicata, cellula basali pedicellata, cellula apicali leniter curvata, 3–5-septata, (39.9–)43.1–51.8–61.4(–63.7) x (1.2–)1.9–2.3–3.4 µm. Clamydosporae absentes.

HOLOTYPUS: Cultura exsiccata in agaro CLA ex MRC 7559, sejuncta a inflorescentis malformatis *Mangifera indica,* Volcani Center, Bet Dagan, Israel, 1993, S. Freeman (PREM 57299).

Colonies on PDA with average growth rate of 3.4 mm/d at 25°C. Aerial mycelium white, floccose. Reverse of colonies sometimes rosy buff (17"f) to dark purple (65k). Conidiophores on aerial mycelium originating erect and prostrate from substrate. Conidiophores sympodially branched bearing mono- and polyphialides (Fig. 1, 2). Polyphialides have 2–5 conidiogenous openings (Fig. 1, 2). Phialides on the aerial

conidiophores mono- and polyphialidic, up to 30.0 µm long and 3 µm wide. Sterile hyphae absent. Microconidia variable in shape, obovoid conidia the most abundant type, oval to allantoid conidia occurring occasionally (Fig. 3). Microconidia mostly 0-septate with 1-septate conidia occurring less abundantly, 0-sepate: (2.5–)4.3–9.0–14.4 x (1.5–)1.7–2.4–3.3 µm. Sporodochia present, cream (19'f) and orange (15b). Macroconidia long and slender, usually 3–5 septate (Fig. 4): (39.9–)43.1–51.8–61.4(–63.7) x (1.2–) 1.9–2.3–3.4 µm. Chlamydospores absent.

Etymology: *Mangiferae* (L. gen) indicating the species association with the genus *Mangifera* L.

Specimens examined: ISRAEL: Bet Dagan, Volcani center plantation. Mango malformation inflorescence on *M. indica*, 1993, *S. Freeman* 506/2 (PREM 57299, HOLOTYPE; MRC 7559, ex-holotype); Ginosar. Inflorescence malformation of *M. indica* cultivar Kent, 1998, *S. Freeman* 34 (MRC 7560); Sde Nitzar. Inflorescence malformation of *M. indica*, 1998, *S. Freeman* 41 (MRC 7561); Bene Dror. Inflorescence malformation of *M. indica* cultivar Keitt, 1998, S. *Freeman* 86 (MRC 7562). SOUTH AFRICA. MPUMALANGA: Nelspruit. Inflorescence malformation of *M. indica,* 1982, *F. Wehner* MRC 2730 (PREM 57300, PARATYPE; KSU 3873, ex-paratype); inflorescence malformation of *M. indica*, 1998, *H. Britz* NS1-1 (MRC 8080); Inflorescence malformation of *M. indica*, 1998, *H. Britz* NS1-9 (MRC 8081); Nelspruit, Fredenheim. Inflorescence malformation of *M. indica*, 1998, *H. Britz* FH1-6 (MRC 8085); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1537, FH1-8 (MRC 8078); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1542, FH1-16 (MRC 8079); inflorescence malformation of *M. indica*, 1998, *H. Britz* FH1-73 (MRC 8084). MPUMALANGA: Malelane. Inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1551 = ML3-1 (MRC 8077); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1547 = ML1-9 (MRC 8082); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1545 = ML1-6 (MRC 8086); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1546 = ML1-8 (MRC 8087); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1548 = ML2-1 (MRC 8083); KWAZULU-NATAL: Inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3477 (PREM 57301, PARATYPE; KSU-X3875, ex-paratype); inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3478

(KSU-X 3876); inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3479 (KSU-X 3877). EGYPT: Inflorescence malformation of *M. indica, Ibrahim Mausour,* KSU-X4706 (MRC 8089); inflorescence malformation of *M. indica, Ibrahim Mausour* KSU-X4702 (MRC 8090); inflorescence malformation of *M. indica, Ibrahim Mausour* KSU-X4700 (MRC 8091). USA. FLORIDA: Miami, Dade County. Inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS16 (MRC 7034); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS23 (MRC 7035); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS55 (MRC 7038); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* MRC 7039; inflorescence malformation of *M. indica*, *R. Ploetz* KSU-X4079 = FRC-M3622 (MRC 8088). MALAYSIA: Inflorescence malformation of *M. indica*, *Baharuddin Salleh* KSU-X4382 (MRC 8092); inflorescence malformation of *M. indica, Baharuddin Salleh* KSU-X4384 (MRC 8093).

Commentary: A dried culture to serve as holotype has been deposited at the Plant Protection Research Institute, Pretoria, South Africa (PREM 57299). Ex-holotype cultures have been deposited in the culture collection of the South African Medical Research Council, Tygerberg, South Africa (MRC 7559) and the department of Plant Pathology, Kansas State University, Manhattan, Kansas, USA (KSU 11781). Ex-paratype cultures have been deposited as MRC 2730 (KSU-X3873) and MRC 3477 (KSU-X3875).

Fusarium sterilihyphosum Britz, Marasas & Wingfield, sp. nov. Figs. 5-8

Coloniae in agaro PDA apud 25°C 4.8 mm per diem crescentes. Mycelium aerium floccosum, subalbum, infra giseo-roseum ad pallido- purpureum. Conidiophora in agaro CLA erecta vel prostrata, simplicia vel ramosa, cellulae conidiogenae mono- et polyphialides, usque ad 30 x 3 µm. Hyphae steriles circinatae praesentes. Microconidia in capitulis falsis, hyalina, plerumque obovoidea, subinde ovata vel allantoidea, plerumque 0-septata, subinde 1-septata, (2.3–)4.5–8.8–24.2 x (1.4–)1.6–2.6–3.5(–3.8) µm. Sporodochia raro praesentia. Sporodochia raro praesentia, alba ad aurantiaca. Macroconidia hyalina, falcata, gracilia, leniter curvata, tenuitunicata, cellula basali pedicellata, cellula apicali leniter curvata, 3-5-septata, (24.5–)28.4–37.1–47.1(–49.7) x (2.3–)2.4–3.2–4.1 µm. Clamydosporae absentes.

HOLOTYPUS: Cultura exsiccata in agaro CLA ex MRC 2802, sejuncta a inflorescentis malformatis *Mangifera indica,* Letsitele, Tzaneen, Northern Province, South Africa, 1982, J. M. Darvas (PREM 57302).

Colonies on PDA with average growth rate of 4.8 mm/d at 25°C. Aerial mycelium almost white ('1). Reverse of colonies straw to grayish rose (3"f) and light purple (63i). Conidiophores on aerial mycelium erect, occasionally prostrate. Conidiophores sympodially branched bearing mono- and polyphialides (Fig. 5). Phialides on aerial conidiophores mono- and polyphialidic, up to 30.0 µm long and 3 µm wide. Sterile hyphae present (Fig. 6). Microconidia obovoid, oval to allantoid, 0-septate conidia abundant, 1-septate conidia less common (Fig. 7, 8): 0-septate: 4.5–8.8–14.2 x 1.6–2.6–3.5 µm. Sporodochia seldom present, cream (19'f) to orange (15b). Macroconidia slightly beaked apical cells, a footlike basal cell, 3-5 septate (Fig. 8), 28.4–37.1–47.1 x 2.4–3.2–4.1 µm. Chlamydospores absent.

Etymology: *Sterilihyphosum* (L. adj) refers to the presence of sterile hyphae in mycelium.

Specimens examined: SOUTH AFRICA. NORTHERN PROVINCE: Tzaneen, Letsitele area. Mango malformation inflorescence on *M. indica,* 1982, *J. M. Darvas* MRC 2802 = NRRL 25623 (PREM 57302, HOLOTYPE; KSU-X3874, ex-holotype); mango malformation of *M. indica,* 1997, *H. Britz* A33-1 (MRC 7606); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1315 = A40-1 (PREM 57303, PARATYPE; MRC 8095, ex-paratype); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1367 = A1-2 (MRC 7602); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1398 = A20-1 (MRC 7605); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1286 = A26-1 (PREM 57304, PARATYPE; MRC 8101, ex-paratype); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1478 = B12-1 (MRC 8102); mango malformation of *M. indica,* 1997, *H. Britz* C6-1 (MRC 8103); mango malformation of *M. indica,* 1997, *H. Britz* D2-1 (MRC 8094); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1146 = E6-1 (MRC 8096); Deerpark. Mango malformation of *M. indica,* 1998, *H. Britz* DP3-5 (MRC 8100); mango malformation of *M. indica,* 1998, *H. Britz* DP3-7 (MRC 8106); mango malformation of *M. indica,*

1998, *H. Britz* FCC 1632 = DP3-9 (MRC 8107). MPUMALANGA: Hazyview. Mango malformation of *M. indica,* 1998, *H. Britz* FCC 1563 = HZ1-9 (MRC 8099); mango malformation of *M. indica,* 1998, *H. Britz* FCC 1555 = HZ1-1 (MRC 8104); mango malformation of *M. indica,* 1998, *H. Britz* FCC 1557 = HZ1-3 (MRC 8108); Malelane. Mango malformation of *M. indica,* 1998, *H. Britz* ML2-10 (MRC 8105).

Commentary: A dried culture to serve as holotype has been deposited at the Plant Protection Research Institute, Pretoria, South Africa (PREM 57302). Ex-holotype culture specimens have been deposited in the culture collection of the South African Medical Research Council, Tygerberg, South Africa (MRC 2802) and the department of Plant Pathology, Kansas State University, Manhattan, Kansas, USA (KSU-X3874). Ex-paratype cultures have been deposited as MRC 8095 (KSU 11783) and MRC 8101 (KSU 11782).

Of the three *Fusarium* species found associated with mango malformation, two have been described as new in this study. The third group included three isolates (KSU-X4379, KSU-X4381 and KSU-X2330) that were collected from malformed mango tissue in Malaysia. These isolates have sparse aerial mycelium. Their aerial mycelial conidiophores emerge directly from the substrate hyphae (referred to as erect). The polyphialides of these isolates have fewer than 3 conidiogenous openings. Microconidia are borne in false heads and are obovoid in shape, predominantly without septa but with 1-septate conidia occurring occasionally. Macroconidia are short 19.3–24.8–29.5 x 1.3–2.0–3.0 µm and 3-5 septate. Chlamydospores and sterile coiled hyphae are absent (Table 2). We believe that this fungus also represents a new taxon, but the collections are insufficient in number to justify a formal description at this stage.

The newly described species, *F. mangiferae* and *F. sterilihyphosum* had different growth rates on PDA at 25°C. *F. mangiferae* had a slower growth rate than *F. sterilihyphosum*, but variation in growth rate among isolates of both species was observed. The one way ANOVA indicated that the growth rate did not differ significantly when the growth rate of all the isolates of both species were analyzed ($P>0.001$). Colony color of the two species was the same.

Mating type and sexual compatibility tests

All isolates of the undescribed *Fusarium* species represented by only 3 isolates were of the *MAT*-1 mating type. Most of the *F. mangiferae* isolates were *MAT*-2 except for two isolates from Malaysia (MRC 8092 and MRC 8093) that were *MAT*-1. Both mating types were identified amongst *F. sterilihyphosum* isolate. The majority of these isolates were *MAT*-1 and isolates MRC 8101, MRC 8104 and MRC 8105 were *MAT*-2.

Isolates of *F. mangiferae* and *F. sterilihyphosum* of opposite mating type were sexually incompatible when crossed within each species and between the two species. None of the other *Fusarium* isolates (Table 1) were sexually compatible with the tester strains of mating population H.

DISCUSSION

In this study we have shown that at least two distinct *Fusarium* spp. are associated with mango malformation symptoms, namely *F. mangiferae* and *F. sterilihyphosum*. *F. mangiferae* was previously shown to be the causal agent of mango malformation and *F. sterilihyphosum* is associated with similar disease symptoms in South Africa. A third taxon was also identified, but our collections are insufficient in number to justify describing the fungus. Furthermore, the fungus does not occur in South Africa, and has not been a primary focus of our investigation.

The results of this study, together with those of Steenkamp *et al.* (2000a), have shown that mango malformation in South Africa is associated with two distinct species, *F. mangiferae* and *F. sterilihyphosum*. *F. sterilihyphosum* has only been isolated from malformed mango tissue in South Africa. The histone *H3* and β-tubulin gene sequences for isolates of *F. mangiferae* are similar to those of *F. subglutinans* strains NRRL 25226 and MRC 7559 (Steenkamp *et al.*, 2000a), which were previously reported to be the causal agent of mango malformation (Freeman *et al.*, 1999).

Fusarium mangiferae has been isolated from mango malformation symptoms in various geographical areas, such as South Africa, Florida, Egypt, India, Israel and Malaysia. *Fusarium mangiferae* is morphologically most similar to *F. concentricum*

Nirenberg & O'Donnell and *F. guttiforme* Nirenberg & O'Donnell. *F. concentricum* has long slender 3–4 septate macroconidia similar to those produced by *F. mangiferae,* which has sympodially branched conidiophores in contrast to the branched conidiophores of *F. concentricum.* *Fusarium guttiforme* can be distinguished from *F. mangiferae* based on the presence of the uniformly obovoid microconidia and 3-septate macroconidia that are shorter in length than those of *F. mangiferae.* The occasional production of 3-septate macroconidia in *F. guttiforme* isolates (MRC 7539, MRC 6784 and MRC 6785) found in the present study was also observed by Viljoen *et al.* (1997). Nirenberg & O'Donnell (1998) did not refer to the macroconidial characteristics in their description of *F. guttiforme.*

Fusarium sterilihyphosum has been isolated only from South Africa. This species is morphologically similar to *F. mangiferae,* but can be distinguished from *F. mangiferae. F. sterilihyphosum* has shorter 3–5 septate macroconidia, faster growth rate on PDA at 25°C than *F. mangiferae* and produces sterile coiled hyphae. *F. sterilihyphosum* is most closely related to *F. guttiforme* based on histone gene sequence (Steenkamp *et al.,* 2000a). Morphologically, *F. sterilihyphosum* resembles *F. circinatum* and *F. pseudocircinatum* O'Donnell & Nirenberg. These three species all produce sterile coiled hyphae. However, macroconidia are long, slender and 3–5 septate in *F. sterilihyphosum,* while shorter 3-sepate macroconidia are produced in both *F. circinatum* and *F. pseudocircinatum.*

Both *F. sterilihyphosum* and *F. mangiferae* are morphologically distinct from species belonging to *F. subglutinans sensu lato* occurring on various host plants, including *F. begoniae* Nirenberg & O'Donnell, *F. bulbicola* Nirenberg & O'Donnell, *F. circinatum* (MP-H), *F. guttiforme, F. concentricum, F. pseudocircinatum, F. sacchari* (Butler) W. Gams (MP-B) and *F. subglutinans sensu stricto* (MP-E) (Table 2). *Fusarium mangiferae* and *F. sterilihyphosum* can also be distinguished from each other based on morphological characteristics. Sterile coiled hyphae and shorter 3–5 septate macroconidia produced by *F. sterilihyphosum* distinguish it from *F. mangiferae. Fusarium mangiferae* had a slower growth rate than *F. sterilihyphosum* on PDA at 25°C. However, growth rate is a secondary morphological characteristic and no significant difference ($P>0.001$) among isolates of both species was observed. Furthermore, secondary characteristics are generally not used in species descriptions

in view of the variability within populations and / or the instability of these characters (Gerlach & Nirenberg, 1982; Nelson *et al.*, 1983; Nirenberg & O'Donnell, 1998).

Both mating types (*MAT*-1 and *MAT*-2) were identified in *F. mangiferae* and *F. sterilihyphosum* isolates. This is in contrast to the Steenkamp *et al.* (2000a) study, where isolates of each of these species included only a single mating type. In the present study, isolates of the two species having opposite mating type were sexually incompatible.

Likewise, isolates of *F. mangiferae* and *F. sterilihyphosum* of different mating types failed to cross with each other. This failure to produce sexual crosses could be explained by sterility, female-sterility of isolates or unfavorable conditions for crosses to occur (Perkins, 1994; Leslie, 1995). At this stage, there is thus no evidence to suggest that sexual outcrossing is occurring within or between these two fungi from mango.

Mango is native to Asia, eastern India, Burma, and the Andaman Islands, and mango malformation was first reported over a century ago in India (Kumar *et al.*, 1993). *F. mangiferae* isolates from South Africa, United States, Israel, Malaysia and Egypt grouped into the so-called 'Asian clade' of O'Donnell *et al.* (1998) based on histone *H3* and β-tubulin gene sequences (O'Donnell *et al.*, 1998; O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a). *F. mangiferae* from different geographical areas was most probably introduced from India (Zheng & Ploetz, 2002), which would explain the presence of *F. mangiferae* isolates grouping in the 'Asian clade'. *F. sterilihyphosum* isolates from mango malformation symptoms in South Africa grouped into the so-called 'American clade' (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a). O'Donnell *et al.* (2000) speculated that *F. sterilihyphosum* (MRC 2802 = NRRL 25623) originated from mango that was imported into South Africa from South America, although the basis for this supposition is not known to us. However, vegetative malformation has been reported in Mexico (Noriega-Cantu *et al.*, 1999). These Mexican isolates produce sterile coiled hyphae and grouped also in the 'American clade' based on β-tubulin gene sequences, like *F. sterilihyphosum* isolates from South Africa (David M. Geiser, personal communication). Clearly, further

investigations with strains from South America would be required to test the hypothesis that *F. sterilihyphosum* isolates in South Africa originated in South America.

The fact that three distinct taxa are found associated with mango malformation symptoms emphasizes a serious problem regarding the etiology of mango malformation disease. *Fusarium mangiferae* has been unequivocally indicated as the causal agent of mango malformation (Freeman *et al.*, 1999). It is, however, not known whether *F. sterilihyphosum* or the undescribed *Fusarium* sp. are also able to cause diseases on mango trees. Their role in the etiology of mango malformation disease clearly requires further intensive study.

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Table 1. *Fusarium* species isolated from malformed mango tissue.

Species	Origin	MRC no[a]	Other no[b]	Original Source[c]
Fusarium sp.	Malaysia	8079	KSU-X4379	B. Salleh
	Malaysia	8070	KSU-X4381	B. Salleh
	Malaysia	8071	KSU-X2330	B. Salleh
F. mangiferae	South Africa (ex-paratype)	2730	KSU-X3873	F. Wehner
	South Africa (ex-paratype)	3477	KSU-X3875	C. Crookes
	South Africa	3478	KSU-X3876	C. Crookes
	South Africa	3479	KSU-X3877	C. Crookes
	South Africa	8077	FCC 1551, ML3-1	H. Britz
	South Africa	8078	FCC 1537, FH1-8	H. Britz
	South Africa	8079	FCC 1542, FH1-16	H. Britz
	South Africa	8080	NS1-1	H. Britz
	South Africa	8081	NS1-9	H. Britz
	South Africa	8082	FCC 1547, ML1-9	H. Britz
	South Africa	8083	FCC 1548, ML2-1	H. Britz
	South Africa	8084	FH1-73	H. Britz
	South Africa	8085	FH1-6	H. Britz
	South Africa	8086	FCC 1545, ML1-6	H. Britz
	South Africa	8087	FCC 1546, ML1-8	H. Britz
	Florida, USA	7034		R. Ploetz
	Florida, USA	7035		R. Ploetz
	Florida, USA	7038		R. Ploetz
	Florida, USA	7039		R. Ploetz
	Florida, USA	8088	KSU-X4079, FRC-M3622	R. Ploetz
	Egypt	8091	KSU-X4700	I. Mausour
	Egypt	8090	KSU-X4702	I. Mausour
	Egypt	8089	KSU-X4706	I. Mausour

Species	Origin	MRC no[a]	Other no[b]	Original Source[c]
F. mangiferae	Israel (ex-holotype)	7559	FCC 73, KSU 11781, 506/2	S. Freeman
	Israel	7560	FCC 74	S. Freeman
	Israel	7561	FCC 80	S. Freeman
	Israel	7562	FCC 81	S. Freeman
	Malaysia	8092	KSU-X4382	B. Salleh
	Malaysia	8093	KSU-X4384	B. Salleh
F. sterilihyphosum	South Africa (ex-holotype)	2802	KSU-X3874, NRRL 25623	J. Darvas
	South Africa	7602	FCC 1367, A1-2	
	South Africa	7605	FCC 1398, A20-1	H. Britz
	South Africa	7606	A33-1	H. Britz
	South Africa	8094	D2-1	H. Britz
	South Africa (ex-paratype)	8095	FCC 1315, KSU 11783, A40-1	H. Britz
	South Africa	8096	FCC 1143, E6-1	H. Britz
	South Africa	8099	FCC 1563, HZ1-9	H. Britz
	South Africa	8100	DP3-5	H. Britz
	South Africa (ex-paratype)	8101	FCC 1286, KSU 11782, A26-1	H. Britz
	South Africa	8102	FCC 1478, B12-1	H. Britz
	South Africa	8103	C6-1	H. Britz
	South Africa	8104	FCC 1555, HZ1-1	H. Britz
	South Africa	8105	ML2-10	H. Britz
	South Africa	8106	DP3-7	H. Britz
	South Africa	8107	FCC 1632, DP3-9	H. Britz
	South Africa	8108	FCC 1557, HZ1-3	H. Britz

[a] MRC = Culture collection of the Medical Research Council, Tygerberg, South Africa.

[b] KSU-X= Kansas State University culture collection, Department of Plant Pathology, Kansas State University, Manhattan. Kansas, USA, FRC = *Fusarium* Research

Center, Pennsylvania State University, USA, NRRL = Northern Regional Research Laboratory, Peoria, Illinois, USA, FCC = *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa with original numbers indicated as follows: A, B, C, D, E = Isolates from different orchards from Letsitele, South Africa, HZ = Isolates from Hazyview, South Africa, ML = Isolates from Malelane, South Africa, DP = Isolates from Deer Park, South Africa, FH = Isolates from Fredenheim farm, Nelspruit, South Africa, NS = Isolates from Nelspruit, South Africa.

[c] B. Salleh collected isolates KSU-X2330, 4379, 4381, 4382 and 4384 in Malaysia; Ibrahim Mausour collected KSU-X4079, 4700, 4702 and 4706 in Egypt; C. Crookes collected isolates MRC 3477–3479 in KwaZulu-Natal, South Africa; S. Freeman collected MRC 7559–7562 in Israel; R. Ploetz collected isolates MRC 7034–7035, 7038–7039 in Florida, USA.

Table 2. Distinguishing characteristics described by Nirenberg and O'Donnell (1998) of isolates of *Fusarium subglutinans sensu lato* as well as characteristics observed for isolates in this study.

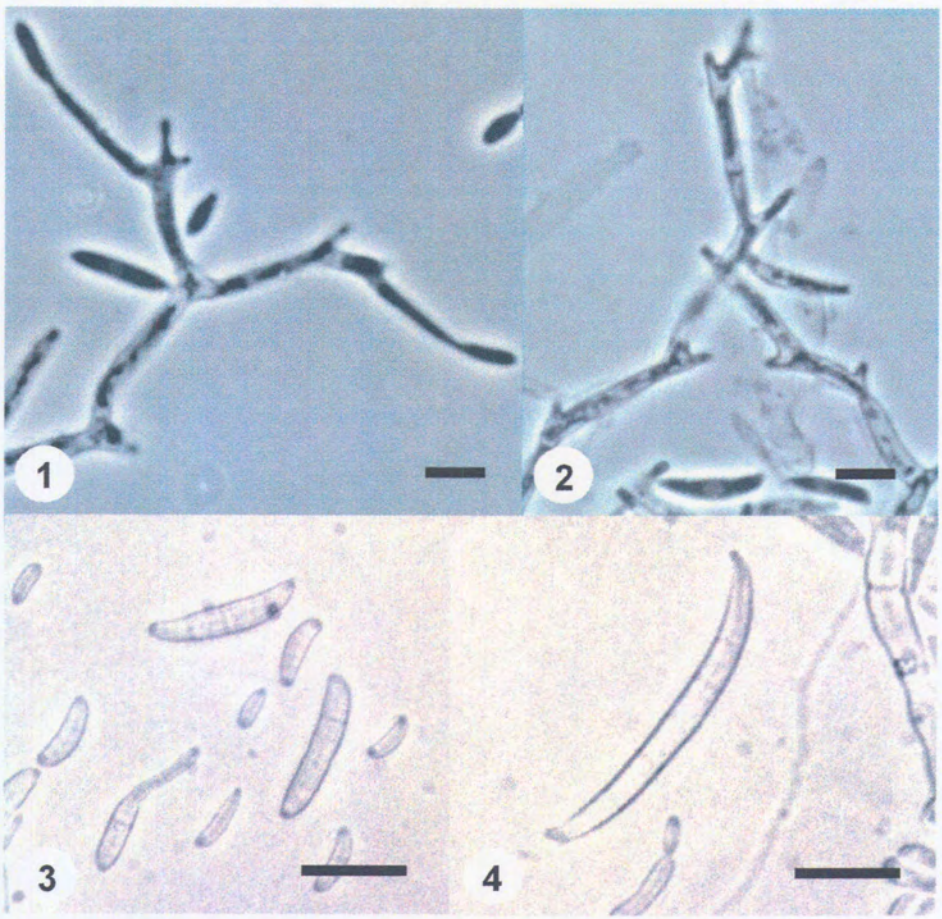
	F. subglutinans sensu lato								New species		
Morphological characteristics[a]	*F. begoniae*	*F. bulbicola*	*F. circinatum*	*F. concentricum*	*F. guttiforme*	*pseudocircinatu*	*F. sacchari*	*F. subglutinans*	*F. mangiferae*	*sterilihyphosum*	Fusarium sp.
Microconidia obovoid	+	+	+	+	+	+	-	-	+	+	+
Microconidia oval to allantoid / or fusoid	+	+	(+)	+	-	+	+	+	+	+	+
Sterile coiled hyphae	-	-	+	-	-	+	-	-	-	+	-
Conidiophore originated erect	-	-	+	+	+	?	-[b]	+[b]	+	+	+
Conidiophore originated prostrate	+	+	-	-	+	+	+[b]	+[b]	+	+	-
Conidiogenous openings: ≤3	+	+	-	-	-	-	-[b]	+	-	-	+
Conidiogenous openings: ≥3	-	-	+	+	+	+	+[b]	-	+	+	-
Microconidia 0-septate, occasionally 1-septate	+	+	+	+	+	+	+[c]	-[c]	+	(+)	+
Microconidia 0-3 septate	-	-	-	-	-	-	-[c]	+[c]	-	-	+
Macroconidia 3-septate	+	-	+	-	+	+	+[c]	-[c]	-	-	-
Macroconidia 3-5 septate	-	+	-	+	-	-	-[c]	+[c]	+	+	+
Host specific	+	+	+	?	+	?	+	+	?	?	?

[a] + indicates the presence of the characteristic, (+) indicates that the character is not present in all isolates of the species, - indicates the absence of the characteristic and ? indicates that the characteristic has not been reported.

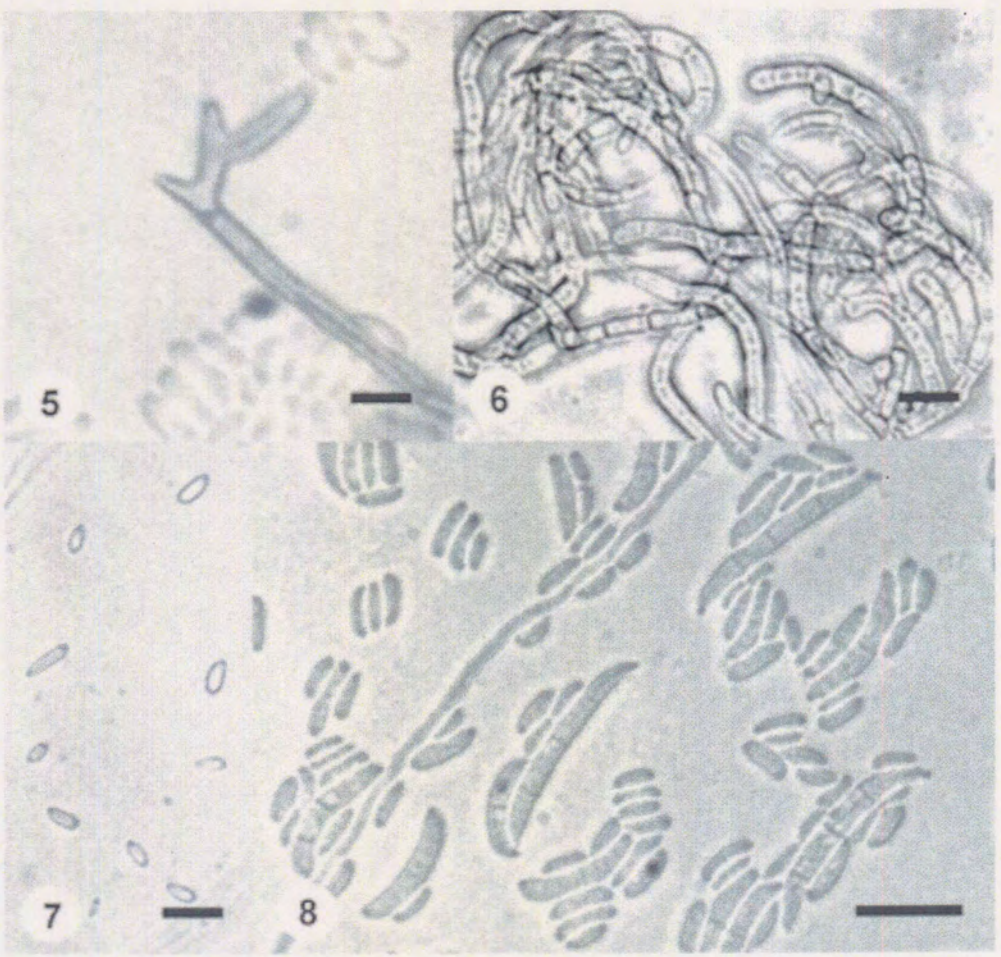
[b] Morphological characteristic identified from *F. sacchari* isolates (FRC-M941, 943) from sugarcane in India (MP-B) and *F. subglutinans* isolates (BBA 11157 from Iran and FRC-M3696 from St. Elmo, Illinois).

[c] Morphological characteristics obtained from Gerlach & Nirenberg (1982).

Fig. 1–4. *Fusarium mangiferae.* 1. Branched conidiophores bearing polyphialides with 3 conidiogenous openings (bar = 5µm). 2. Branched conidiophores bearing mono- and polyphialides (bar = 5µm). 3. Microconidia (bar = 15µm). 4. Macrocondium (bar = 15µm).



FIGS. 5–8. *Fusarium sterilihyphosum.* 5. Conidiophores bearing polyphialides with 3 conidiogenous openings (bar = 5µm). 6. Sterile coiled hyphae (bar = 10µm). 7. Microconidia (bar = 10µm). 8. Microconidia with 0–1 septa and 3-septate macroconidia (bar = 20µm).



CHAPTER 7

VEGETATIVE COMPATIBILITY AND DISTRIBUTION OF TWO *FUSARIUM* SPECIES ASSOCIATED WITH MANGO MALFORMATION IN SOUTH AFRICA

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ABSTRACT

Mango malformation is an economically important disease of mango in many parts of the tropics and sub-tropics. The causal agent of this disease has recently been described as *Fusarium mangiferae* (=*F. subglutinans sensu lato*). In South Africa, *F. mangiferae* and a second species, known as *F. sterilihyphosum,* are associated with malformed mango tissue. In this study, the distribution and vegetative compatibility of *F. mangiferae* and *F. sterilihyphosum* isolates collected in South Africa were determined. In South Africa, *F. sterilihyphosum* and *F. mangiferae* were found to have distinct distributions with the former species primarily occurring in the Northern Province and the latter in Mpumalanga and KwaZulu-Natal. Based on vegetative compatibility, isolates of both species represent a single clone. From these results, it is evident that both species are associated with mango malformation symptoms and that they represent single genets in each of the areas where they occur.

INTRODUCTION

Mango malformation is one of the most important diseases affecting mango (*Mangifera indica* L.) production in the tropics and sub-tropics. The disease has been reported from all major mango-growing areas including Egypt, Brazil, Florida, India, Israel, Mexico Pakistan and South Africa (Varma, 1983; Kumar *et al.*, 1993; Ploetz, 1994a). Mango malformation induces abnormal development of vegetative shoots and inflorescences. Of these, inflorescence malformation is the most dominant symptom causing abnormal thick fleshy blossoms on profusely branched panicles (Varma, 1983; Kumar *et al.,* 1993; Ploetz, 1994a).

The fungal pathogen identified as *Fusarium subglutinans* (Wollenweber & Reinking) Nelson *et al.* (= *F. moniliforme* Sheldon var. *subglutinans* Wollenweber & Reinking) has been shown to be associated with mango malformation (Summanwar *et al.*, 1966). There has been considerable dispute regarding the cause of mango malformation (Varma, 1983; Kumar *et al.,* 1993). However, recent pathogenicity tests, where typical mango malformation symptoms were induced with an isolate identified as *F. subglutinans,* have dispelled doubt regarding the cause of the disease (Varma *et al.,* 1974; Manicom, 1989; Ploetz & Gregory, 1993; Freeman *et al.,* 1999).

The fungus primarily responsible for mango malformation in most parts of the world has recently been described as *F. mangiferae* Britz, Wingfield & Marasas (Britz *et al.*, 2002). The justification for this description arises from histone *H3* and β-tubulin gene sequences (Steenkamp *et al.*, 2000) and distinct morphological characteristics (Britz *et al.*, 2002). The latter authors have also shown that a second species in the *F. subglutinans sensu lato* complex is commonly associated with malformation in South Africa. This fungus has been described as *F. sterilihyphosum* Britz, Wingfield & Marasas, although its role as a causal agent of malformation is not yet known.

Vegetative compatibility groups (VCGs) are relatively easy to detect and can provide useful insights into the genetic diversity of different *Fusarium* (section *Liseola*) populations (Leslie, 1995; Leslie & Mansuetus, 1995; Gordon *et al.*, 1996). Despite this, very little attention has been given to the population structure of the mango malformation pathogen, *F. mangiferae,* in various parts of the world. Previous studies in Florida (Ploetz & Gregory, 1993; Ploetz, 1994b) and recently in Egypt, Israel, South Africa and USA (Zheng & Ploetz, 2002) have shown that populations of *F. mangiferae* are homogeneous in these countries, where most of the isolates belonged to a single dominant VCG.

The aim of this study was to determine the distribution as well as the VCG diversity of the two *Fusarium* spp. associated with mango malformation in South Africa. To achieve this goal, we collected inflorescence tissue from mango-growing areas in South Africa. We then used morphology and ITS-RFLPs, which were developed as part of the present study, to distinguish isolates of *F. sterilihyphosum* and *F. mangiferae* from each another. The distribution of the two fungi and the genetic diversity of their populations in South Africa was then determined.

MATERIALS & METHODS

Morphological and cultural studies

In South Africa, *Fusarium* spp. were isolated from malformed blossoms collected in 1997 and 1998 in Letsitele (23'50''S 30'09''E), Trichardtsdal and Deerpark (Northern Province). Isolates were also collected from Nelspruit (25'28''S 30'58''E),

Fredenheim farm near Nelspruit, Malelane (25'30''S 31'28''E) and Hazyview (Mpumalanga) (Fig. 1). An isolate associated with mango malformation in Florida (MRC 7035) was received from R. Ploetz. The South African isolates received from the Medical Research Council (MRC) culture collection included: isolate MRC 2802 (NRRL 25623) collected by J. Darvas in 1982 in the Letsitele area, Tzaneen, Northern Province, isolate MRC 2730 collected in 1982 by F. Wehner in Nelspruit, Mpumalanga and isolates MRC3477-3479 collected by C. Crookes in 1984 in KwaZulu-Natal (Britz *et al.*, 2002). All isolates were stored in 15% glycerol at –70 °C in the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and representative isolates of *F. mangiferae* and *F. sterilihyphosum* (Table 1) have been deposited in the culture collection of the Medical Research Council (MRC), South Africa.

Fusarium spp. were isolated in South Africa from mango inflorescence clusters showing typical symptoms of blossom malformation. A selective medium (Nash & Snyder, 1962) was used to facilitate isolation. Single conidial isolates were prepared and stored in 15 % glycerol at –70 °C.

For morphological observations, all isolates were grown on CLA (Fisher *et al.*, 1982) and KCl agar (Nelson *et al.*, 1983) for 10 to 14 days at 23°C under near-ultraviolet light and cool-white light with a 12-hour photoperiod. *Fusarium* spp. were identified using morphological characteristics described by Nirenberg & O'Donnell (1998) and Britz *et al.* (2002).

ITS-RFLP

Previous studies have shown that a *F. sterilihyphosum* (MRC 2802 = NRRL 25623) isolate and *F. mangiferae* (NRRL 25226, and 506/2 = MRC 7559) isolates have different major ITS2 types (Freeman *et al.*, 1999; O'Donnell *et al.* 1998; 2000). We, therefore, used this difference as the basis to develop a restriction fragment length polymorphism (RFLP) technique to rapidly confirm the identity of *F. mangiferae* and *F. sterilihyphosum* isolates. The ITS regions of additional isolates of both species were sequenced in order to develop this technique.

DNA extraction

Fusarium mangiferae and *F. sterilihyphosum* isolates of were grown on complete medium agar (CMA) (Correll *et al.,* 1987) for 10 days. Mycelium was scraped from the CMA plates, frozen in liquid nitrogen and crushed to a fine powder with a mortar and pestle. The powdered mycelium was transferred to 1.5 ml Eppendorf™ tubes and DNA was extracted as described by Raeder & Broda (1985).

PCR amplification and sequencing

Amplification of the ITS1, 5.8S and ITS2 rDNA regions was achieved using primers ITS 1 and 4 (White *et al.,* 1990). PCR reactions were performed in 50 µl containing 1 µM of both primers, 0.1 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, and 1U of *Taq* DNA polymerase (Roche Diagnostics, Germany). PCR reactions were performed using an Omnigene thermocycler (Hybaid, Middlesex, United Kingdom) with an initial denaturing step of 1 min at 94°C. This step was followed by 35 cycles of 1 min at 92°C, 1 min at 56°C and 1 min at 72°C. A final extension was performed at 72°C for 5 min.

PCR products were purified with the High Pure™ PCR product purification kit (Roche Diagnostics, Germany). Both strands of the ITS1, 5.8S and ITS2 regions of isolates MRC 2802, MRC 7602, MRC 7035 and MRC 3477 were sequenced. Reactions were performed on an ABI Prism 377 DNA sequencer using the BigDye terminator cycle sequencing kit (Perkin Elmer Applied BioSystems). DNA sequences from isolates MRC 3477, MRC 7035 and MRC 7602 that were sequenced in this study were deposited in the Genbank database with accession numbers AF430128, AF430129 and AF430130, respectively. These sequences were also aligned manually with sequences AF158305 (MRC2802 = NRRL 25623) and U61691 (NRRL 25226) obtained from the Genbank database (aligned sequences in Appendix 2). The ITS2 sequences of isolates MRC 2802, MRC 7035 and MRC 3477 were identified as either ITS2 type I or type II by comparing the sequences with those in the Genbank database (O'Donnell *et al.*, 1998; 2000).

Restriction digests

Restriction sites that distinguished between ITS2 type I and type II were determined with Webcutter 1.0 (RE analysis). Digests were performed on 63 isolates from South Africa using the restriction enzyme, *Alu* I, in a total reaction volume of 20 µl that contained 15 µl of PCR product and 5 U of the enzyme. All digestion reactions were incubated at 37°C for 6 h. Digested PCR fragments were separated using 2% (w/v) agarose gels (Promega Corporation, Madison, Wisconsin, USA) containing ethidium bromide (0.1 µg/ml) and visualized using an ultraviolet transilluminator (Ultra-Violet Product).

Vegetative compatibility tests

Nitrate non-utilizing (*nit*) mutants were generated on 1.5 – 2.5% chlorate agar and the *nit* mutants were identified as *nit1*, *nit3* or nitM based on the utilization of different nitrogen sources (Puhalla & Spieth, 1983; 1985; Correll *et al.*, 1987). Incompatibility was determined by pairing isolates in all possible combinations on minimal media for 14 days at 25°C. Heterokaryon self-incompatibility (HSI) of isolates (Correll *et al.*, 1989) was determined by crossing a *nit1* or *nit3* mutant with a nitM mutant of the same isolate. All parings were repeated at least once.

RESULTS

Morphology

Fifty-eight *Fusarium* isolates collected from malformed mango tissue in South Africa during 1997 and 1998 were identified based on morphology. In addition, *Fusarium* isolates associated with mango malformation received from colleagues from Florida (1 isolate) and South Africa (5 isolates) were identified using morphology. Thus, 18 of the 58 South African isolates were identified as *F. mangiferae* and the remaining 40 isolates represented *F. sterilihyphosum.* All isolates received from Florida (MRC 7035) and South Africa (MRC 2730, MRC 3477, MRC 3478 and MRC 3479) were identified as *F. mangiferae,* except for a South African isolate MRC 2802 that represents *F. sterilihyphosum* as was shown by Britz *et al.* (2002).

ITS-RFLP

Amplication of the ITS1, 5.8S and ITS2 region of the rDNA operon of *Fusarium* isolates (Table 1) from South Africa resulted in a 450 bp product when amplified with primers ITS1 and ITS 4. The PCR products of three isolates, MRC 2802, MRC 7602, MRC 7035and MRC 3477 were sequenced and aligned with published sequences of both species (O'Donnell *et al.,* 1998; 2000). The sequence of isolate MRC 2802 studied in our laboratory was identical to the that published by O'Donnell *et al.* (2000) in Genbank (AF158305). The restriction enzyme, *Alu* I, was identified from sequence data as being useful to distinguish major ITS2 type I and II isolates from one another. These sequence data showed that *F. sterilihyphosum* had the ITS2 type I, including two *Alu* I restriction sites and *F. mangiferae* has the ITS2 type II, without *Alu* I restriction sites. Forty-one of the 63 isolates (Table 1) had the ITS2 type I PCR-RFLP profile producing three RFLP fragments of approximately 380, 50 and 30 bp when digested with *Alu* I (Fig. 2). However, the two smaller fragments (50 and 30 bp) were not visible on a 2% agarose gel. The remaining 22 had the ITS2 type II PCR-RFLP profile producing a single band of approximately 450 bp (Fig. 2).

Vegetative incompatibility tests

Nit mutants were generated for 22 *F. mangiferae* isolates including those from South Africa. Vegetative compatibility tests showed that South African *F. mangiferae* isolates included 2 VCGs. *Nit* mutants were also generated for the 41 *F. sterilihyphosum* isolates (Table 1), which belonged to a single VCG. Two isolates, FCC 1370 and 1525, were heterokaryon self-incompatible (Table 1).

Distribution of Fusarium spp. in South Africa

Isolates collected in the Mpumalanga and Kwazulu-Natal provinces of South Africa were identified as *F. mangiferae*. *F. sterilihyphosum* was isolated from the Northern Province in a 100 km radius around the Letsitele area near Tzaneen (Fig. 1). This species was also isolated from Mpumalanga in the Hazyview area that lies on the border of Mpumalanga and Northern Province (Fig. 1). A single *F. sterilihyphosum* isolate was collected from an orchard in the Malelane area in Mpumalanga.

DISCUSSION

Results of this study have shown that the morphologically distinct *F. mangiferae* and *F. sterilihyphosum* could easily be identified using RFLP profiles from their respective ITS regions. Although this was not a primary objective of the study, this approach will be useful in future studies where cultures originating from mango malformation symptoms can easily be identified.

In this study we have shown that *F. sterilihyphosum* isolates belong to a single VCG and *F. mangiferae* isolates from South Africa belong to two VCGs. Zheng & Ploetz (2002) have suggested that *F. mangiferae* has spread as a clonal population from India to Egypt as well as from Florida to South Africa. It is, therefore, possible that mango malformation caused by *F. mangiferae* in South Africa was introduced from Florida. This view is supported by the fact that several Florida *M. indica* cultivars are grown in South Africa (Zheng & Ploetz, 2002). The limited number of clones of these fungi in South Africa also supports the view that *F. mangiferae* and *F. sterilihyphosum* are exotic pathogens in the country.

Fusarium mangiferae has a wide distribution globally and the fact that it causes mango malformation is now well established (Freeman *et al.*, 1999). *F. sterilihyphosum* is the only species isolated from malformed mango tissue in the Northern Province of South Africa. Furthermore, its consistent association with malformation symptoms suggests strongly that it causes the disease in these areas. Despite this strong anecdotal evidence suggesting that *F. sterilihyphosum* is able to cause mango malformation, pathogenicity remains to be proven.

In South Africa, *F. sterilihyphosum* isolates were found only in the Northern Province. *F. mangiferae* was primarily isolated from Mpumalanga in the Malelane area, Nelspruit area, Fredenheim farm near Nelspruit as well as from Kwazulus-Natal. These findings suggest that *F. mangiferae* and *F. sterilihyphosum* have discrete distributions in South Africa. Although the origin of *F. sterilihyphosum* is not known, it is assumed that it was introduced into South Africa and the distribution of the two fungi is presumably related to movement of mango planting stock in the country.

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Table 1. Origin and vegetative compatibility groups of *Fusarium* isolates associated with mango malformation.

Origin	Strain no.[a]	VCG[b]
F. mangiferae		
South Africa, Nelspruit	MRC 2730	SA1
South Africa, Kwazulu-Natal	MRC 3477, MRC 3478, MRC 3479	SA1
South Africa, Fredenheim	MRC 8078, MRC 8079, MRC 8084, MRC 8085, FCC 1533, FCC 1534, FCC 1538, FCC 1539	SA2
South Africa, Nelspruit	MRC 8080, FCC 1526, FCC 1530, FCC 1532	SA2
South Africa, Malelane	MRC 8077, MRC 8081, MRC 8082, MRC 8083, MRC 8086, MRC 8087	SA2
Florida	MRC 7035	-
F. sterilihyphosum		
South Africa, Letsitele	MRC 2802, MRC 7602, MRC 7606, MRC 7605, MRC 7875, MRC 7877, MRC 8094, MRC 8095, MRC 8101, MRC 8102, MRC 8103	FS1
South Africa, Letsitele	FCC 1370, FCC 1525	HSI
South Africa, Deerpark	MRC 8100, MRC 8106, MRC 8107, FCC 1598, FCC 1604, FCC 1620, FCC 1625, FCC 1629	FS1
South Africa, Hazyview	MRC 8099, MRC 8104, MRC 8108, FCC 1233, FCC 1568, FCC 1570, FCC 1574, FCC 1584, FCC 1585, FCC 1592, FCC 1593, FCC 1594	FS1
South Africa, Trichardtsdal	FCC 1217, FCC 1227, FCC 1228, FCC 1231, FCC 1232, FCC 1233, FCC 1245	FS1
South Africa, Malelane	MRC 8105	FS1

[a] MRC = Culture collection of the Medical Research Council (MRC), Tygerberg, South Africa; KSU-X= Kansas State University culture collection, Department of Plant Pathology, Kansas State University, Manhattan. Kansas; FCC = *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[b] VCG = Vegetative compatibility group, HSI = Heterokaryon self-incompatible.

Fig. 1. Detailed map of sampled area in Mpumalanga and Northern Province.

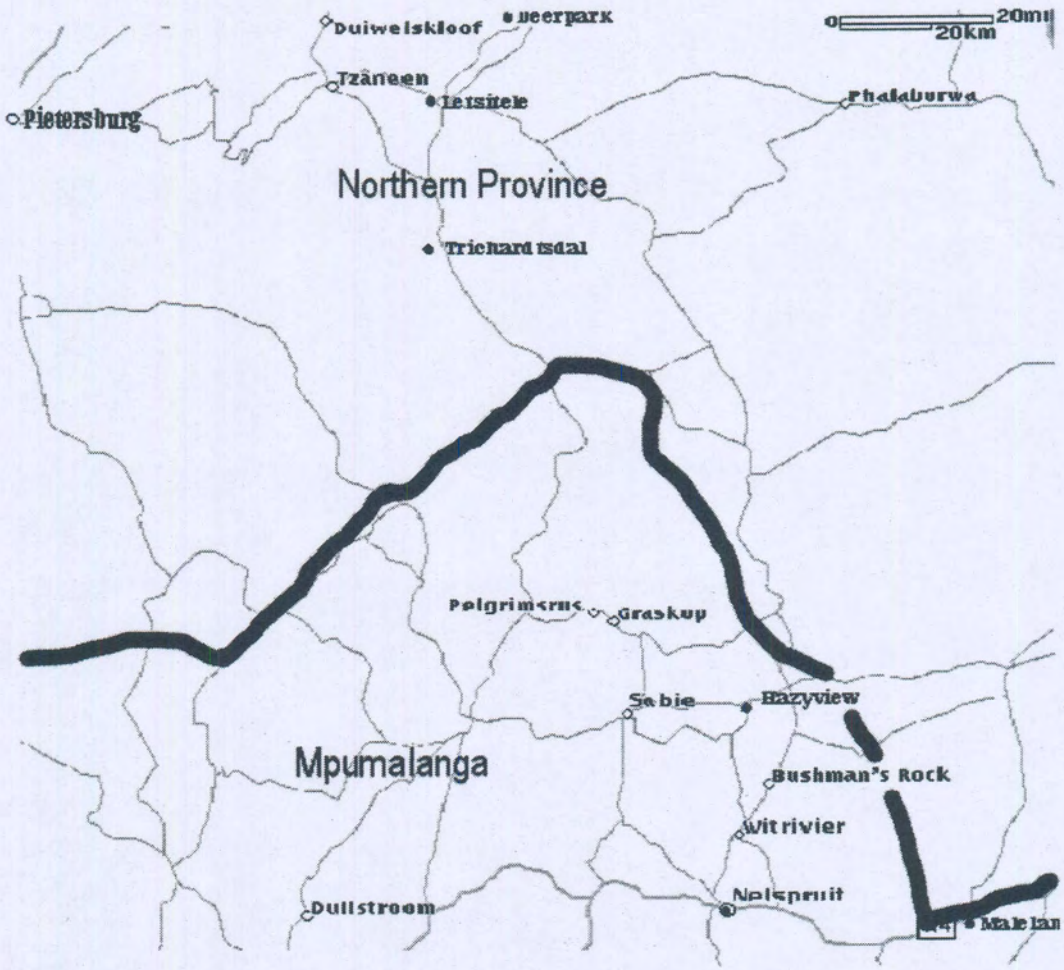

Fig. 2. ITS-RFLP profiled generated by digestion of ITS1, 5.8S and ITS2 rDNA gene with *Alu* I. Lane M, 100 bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp); Lane 1 and 6, *F. sterilihyphosum* isolates MRC 8106 and MRC 8101; Lane 2-5, *F. mangiferae* isolates MRC 7569, MRC 8081, MRC 8085 and MRC 8086.